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Plasmid double locus sequence typing for IncHI2 plasmids, a subtyping scheme for the characterization of IncHI2 plasmids carrying extended-spectrum β-lactamase and quinolone resistance genes

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Objectives: IncHI2 plasmids are frequently encountered in clinical enterobacterial strains associated with the dissemination of relevant antimicrobial resistance genes. These plasmids are usually >250 kb, and technical difficulties can impair plasmid DNA purification and comparison by restriction fragment length polymorphism. We analysed the available IncHI2 whole DNA plasmid sequences to devise a rapid typing scheme to categorize the members of this plasmid family into homogeneous groups.

Methods: We compared the available full IncHI2 plasmid sequences, identifying conserved and variable regions within the backbone of this plasmid family, to devise an IncHI2 typing method based on sequence typing and multiplex PCRs. A collection of IncHI2 plasmids carrying extended-spectrum β-lactamase and quinolone resistance genes, identified in strains from different sources (animals and humans) and geographical origins, was tested by these typing systems.

Results: We devised a plasmid double locus sequence typing (pDLST) scheme and a multiplex PCR discriminating IncHI2 plasmid variants. These systems were tested on a collection of IncHI2 plasmids, demonstrating that the plasmids carrying $bla_{CTX\text{-}M\text{-}2}$ and $bla_{CTX\text{-}M\text{-}9}$ belonged to two major plasmid variants, which were highly conserved among different enterobacterial species disseminated in several European countries.

Conclusions: The ability to recognize and subcategorize plasmids by pDLST in homogeneous groups on the basis of their phylogenetic relatedness can be helpful to analyse their distribution in nature and to discover of their evolutionary origin.

Keywords: CTX-M, pDLST, incompatibility, ESBLs, *qnr*

Introduction

The identification and classification of plasmids in families on the basis of phylogenetic relatedness allows their categorization in homogeneous groups. Molecular comparison of plasmids belonging to the same group can be helpful to describe their evolution by the acquisition or loss of specific genetic determinants, and to trace the emergence and spread of particularly successful plasmids among epidemiologically unrelated bacterial pathogens. For instance, it is interesting to compare plasmids identified in isolates from animal sources with those spreading in human bacterial pathogens, with the aim of investigating a potential animal reservoir of horizontally transmitted resistance genes.

Several phenotypic and molecular methods have been developed for assigning plasmids to families, including the incompatibility grouping by conjugation by Hedges and Datta,[1,2] the Southern blot hybridization method by Couturier *et al.*,[3] and PCR-based replicon typing (PBRT).[4–6] To date, >1000 plasmids have been typed and assigned to specific plasmid families by these methods, detecting some of the major plasmid families occurring in the Enterobacteriaceae (HI2, HI1, I1-γ, X, L/M, N, FIA, FIB, FIC, W, Y, P, A/C, T, K, B/O, FII, U, R, ColE and Q).[4–6]

The most accurate method to characterize a plasmid is based on the determination of its full-length DNA sequence and, to date, five whole IncHI2 plasmids have been sequenced: the reference R478 plasmid from *Serratia marcescens* isolated in 1969 in the USA;[7] pAPEC-O1-R from *Escherichia coli* of avian origin, isolated in the USA in 2006;[8] pK29 identified in a *Klebsiella pneumoniae* from Taiwan in 2007;[9] and pEC-IMP and pEC-IMPQ identified in *Enterobacter cloacae* from Taiwan in 2009.[10] The sequenced IncHI2 plasmids are characterized by specific resistance genes embedded in mobile genetic elements (transposons and insertion sequences), which

constitute the variable part of these plasmids, being also subjected to frequent events of acquisition, loss and rearrangements of genetic material, as previously described.[8,10–12] For instance, pEC-IMP and pEC-IMPQ both carried the bla_{IMP-8} gene conferring carbapenem resistance, but pEC-IMPQ also acquired the *qnrB2* gene conferring reduced susceptibility to fluoroquinolones; the pK29 plasmid conferred resistance to new generation cephalosporins by the presence of the bla_{CMY-8} and $bla_{CTX-M-3}$ genes.[9,10] Neither β-lactamase nor *qnr* genes were reported on the R478 or pAPEC-O1-R plasmids, likely representing the precursors of the IncHI2 plasmids, before the acquisition of these specific resistance genes.[7,8]

Besides the fully sequenced plasmids, a relevant number of IncHI2 plasmids associated with bla_{CTX-M}, bla_{SHV}, bla_{IMP}, bla_{VIM}, *armA*, *qnrA1*, *qnrS1* and *qnrB2* resistance genes have been identified in *Citrobacter youngae*, *E. cloacae*, *E. coli*, *K. pneumoniae*, *Klebsiella oxytoca* and *Salmonella enterica* isolates, becoming the fifth most common plasmid family occurring in the Enterobacteriaceae.[12–14] Furthermore, IncHI2 plasmids are distinguished by their property of optimal transfer by conjugation at temperatures between 22 and 30°C, likely contributing to the spread of resistance in the environment.[7,9,10,13,15,16] The stable permanence of the IncHI2 plasmids might be explained by the presence of several functions providing additional advantages, such as the mutagenesis induction system (*mucAB*), the *relE/relB* toxin–antitoxin system, bacteriophage inhibition (*phi*), and genes conferring resistance to colicin, tellurite, arsenic, copper, silver, mercury and antibiotics.[8,17–19]

The IncHI2 plasmids have been detected as large molecular weight plasmids by S1-PFGE experiments.[20–22] These plasmids normally show a very high molecular weight (>250 kb) and their size often impairs the preparation of good quality plasmid DNA, required for transformation or cloning experiments and comparative analysis by standard methodology, i.e. by restriction fragment length polymorphism (RFLP) analysis. In order to better establish the phylogenetic relatedness of IncHI2 plasmids circulating worldwide, an easier subtyping scheme for IncHI2 plasmids by plasmid double locus sequence typing (pDLST) is herein proposed and applied to a representative collection of IncHI2 plasmids obtained from strains from three continents.

Materials and methods

Comparative analysis of the IncHI2 plasmid scaffold

An *in silico* comparative analysis of the IncHI2 plasmid backbones was performed among the available fully sequenced IncHI2 plasmids: R478 (NCBI reference sequence: NC_005211); pAPEC-O1-R (NC_009838); pK29 (NC_010870); pEC-IMP (NC_012555); and pEC-IMPQ (NC_012556), by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) software. DNA sequences were also compared with the complete DNA sequence of the reference R478 plasmid by two sequence alignments using the Blastnt–Blast2 algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The regions of dissimilarity among the fully sequenced IncHI2 plasmids were further analysed by manual inspection.

pDLST

pDLST was set up on the basis of nucleotide divergence among IncHI2 plasmid backbones. On the basis of the *in silico* analysis, two loci—*smr0018* and *smr0199* in R478, located in the conserved region of these plasmids—were selected for the pDLST. The *smr0018* and *smr0199* chosen primers produced amplicons of 1118 and 536 bp, respectively (Table 1). Total DNA extractions were obtained by the Wizard Genomic DNA Purification System (Promega, Madison, WI, USA). PCR amplifications were performed with the following amplification scheme: 1 cycle of denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and elongation at 72°C for 1 min. The amplification was concluded with an extension of 1 cycle at 72°C for 5 min. PCR products were purified by using the Wizard PCR preps DNA purification system (Promega, Madison, WI, USA), and directly sequenced, using only the forward primer, by fluorescent dye-labelled dideoxynucleotides and a 373 automatic DNA sequencer (Perkin-Elmer, Foster City, CA, USA). The 330 and 460 bp sequences [Figure S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)] were selected within the 1118 bp *smr0018* locus and the 536 bp *smr0199* locus, respectively, for allele assignment. Nucleotide sequences were aligned using DNAMAN software (Lynnon BioSoft, Vaudreuil, Quebec, Canada) set for dynamic full alignment with a gap open penalty of 10, a gap extension of 5, a DNA transition weight of 0.5 and a delay divergent sequence percentage of 30. Allele sequences were submitted at the multilocus sequence typing (MLST) web site www.pubmlst.org/plasmid/ obtaining the respective allele and sequence type (ST) assignments. The prototype sequence alleles identified in this study were submitted to GenBank and released

Table 1. Primers used for the pDLST and HI2 multiplex PCR

Primer name	Sequence	Position nt	Accession no.	Locus target	Amplicon size (bp)/allele size (bp)
pDLST					
smr0018 Fw	5′-ATA ATG ATT CAC CGG GGT AG-3′	18738–18757	NC_005211	*smr0018*	1118/330
smr0018 Rv	5′-CTT CAG GCT ATC GTT TCG-3′	19855–19838			
smr0199 Fw	5′-TGT TTA CAC CAC CAG CAG-3′	179084–179101	NC_005211	*smr0199*	536/460
smr0199 Rv	5′-TTT AAC AAC AGG AGT CGG G-3′	179619–179601			
Multiplex PCR					
hipA Fw	5′-GGA GAC ATT GCT ACC GGG-3′	95049–95066	NC_010870	*hipA*	821
hipA Rv	5′-CGC TAA CAC GTT CAA TGA C-3′	95869–95851			
smr0092 Fw	5′-CTA TGT AAG CAA TGA TCC TC-3′	88861–88880	NC_005211	*smr0092*	499
smr0092 Rv	5′-ACC ATC GGT GTT GAA ATG TG-3′	89359–89340			
smr0183 Fw	5′-GTA TAG AAA GGT TTT AGC TCT-3′	164495–164515	NC_005211	*smr0183*	295
smr0183 Rv	5′-CCG GTT CTT AGG ATC ATC A-3′	164789–164771			

under the accession numbers: GU065228 (smr0018-2); GU065229 (smr0199-1); GU065230 (smr0018-1); and GU065231 (smr0199-2).

Results obtained by pDLST were also confirmed by EcoRI restriction analysis and Southern blot hybridizations performed on representative strains. Briefly, total DNA extractions were obtained by the Wizard Genomic DNA Purification System (Promega). Extracted DNAs were digested with EcoRI and the restriction fragments were separated by 0.8% agarose gel electrophoresis. Gels were transferred onto positively charged nylon membrane (Roche Diagnostics, Monza, Italy) by standard methods[23] and hybridized with digoxigenin-labelled probes for the repHI2 replicon, *smr0018* and *smr0199* regions (digoxigenin PCR DIG Probe Synthesis kit, Roche Diagnostics GmbH, Mannheim, Germany).

IncHI2 plasmid analysis by multiplex PCR

In order to further characterize the IncHI2 plasmids, a multiplex PCR was set up to discriminate the HI2 plasmids assigned to sequence types ST1 and ST2 by pDLST. These PCRs were devised for rapid screening for insertions/deletions characterizing several of the sequenced HI2 plasmids. In particular, three genes—*hipA*, *smr0092* and *smr0183*—targeting the major discrepant regions were selected for this multiplex PCR. Primers for the *hipA*, *smr0092* and *smr0183* amplification are listed in Table 1. The HI2 multiplex PCR amplification was performed with the following amplification scheme: 1 cycle of denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and elongation at 72°C for 1 min. The amplification was concluded with an extension of 1 cycle at 72°C for 5 min. Amplicons were analysed by 2% agarose gel electrophoresis.

Results and discussion

Comparative in silico analysis of fully sequenced IncHI2 plasmids

An *in silico* comparative analysis of nucleotide sequences and their respective encoded open reading frames was performed for five fully sequenced IncHI2 plasmids [Table 2 and Table S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. The backbone of the IncHI2 plasmids was defined as the conserved part present in all the compared sequences and covered >63% of their sequence length (~172/274 kb for R478). This analysis revealed that four out of five IncHI2 plasmids (pK29, pEC-IMP, pEC-IMPQ and R478) showed a high level of conservation of the backbone (nucleotide homology ranging from 99.98% to 99.91%). The pAPEC-O1-R backbone was more divergent (nucleotide homology from 99.17% to 97.04%; Tables 2 and S1). The backbone consisted of four major conserved regions (C1–C4 in Table 2). The conserved backbone of IncHI2 plasmids comprised the replicon IncHI2, the partition system, the conjugative apparatus and the telluric resistance cluster, and also encoded a large number of hypothetical proteins whose function is still undefined. On the basis of the *in silico* analysis, we screened several loci along the plasmid scaffold, which were present in all the IncHI2 plasmids and accumulated significant nucleotide divergence to be used for a plasmid MLST approach. We lastly focused the study on two loci—*smr0018* and *smr0199*—that showed significantly different sequences to be used to discriminate the IncHI2 plasmids. Further loci did not significantly increase the discriminatory power of the sequence-based typing, since, to date, the currently known IncHI2 plasmids show very well-conserved backbones belonging to two major types, one related to R478 and one to pAPEC-O1-R. A pDLST was devised on the two selected loci, targeting the conserved C1 and C3 regions. The *smr0018* gene encodes an open reading frame similar to the *Salmonella typhi* putative surface exclusion protein and *smr0199* encodes a bundle-forming pilus biogenesis protein. By pDLST, the *smr0018* and *smr0199* alleles from plasmids R478, pK29, pEC-IMP and pEC-IMPQ defined the sequence type ST1. The pAPEC-O1-R, showing 13 mismatches in the 330 bp *smr0018* allele sequence and 8 mismatches in the 460 bp *smr0199* allele sequence (Figure S1), was assigned to ST2 (Table 3).

Besides the conserved regions, the comparative analysis revealed larger differences among the IncHI2 plasmids, mostly due to deletions or the acquisition of additional genetic determinants (transposons, insertion sequences, antimicrobial resistance clusters, transporters and toxin/antitoxin systems). These large-scale variations represented the peculiarity of each fully sequenced IncHI2 plasmid. These variable parts can be useful to compare an unknown IncHI2 plasmid with those fully sequenced, to better discriminate the variants belonging to the major ST1 and ST2. For this purpose, we devised a multiplex PCR targeting three variable genetic determinants. In particular, we selected: *hipA*, a hypothetical capsule biosynthesis gene, which is present in pK29, pEC-IMP, pEC-IMPQ and pAPEC-O1-R, but absent in R478; *smr0092*, coding for a hypothetical protein, which is present in R478, pK29, pEC-IMP and pEC-IMPQ, but absent in pAPEC-O1-R; and *smr0183*, coding for a hypothetical lipoprotein, which is present in R478 and pK29, but absent in the other IncHI2 plasmids. These loci were used for the further discrimination of IncHI2 plasmids from our collections. Further PCRs targeting other variable regions can be devised on the basis of the encountered differences (Table S1), to increase the discriminatory power of the comparative analysis among IncHI2 plasmids.

Molecular typing of IncHI2 plasmids, carrying ESBL or quinolone resistance genes

A collection of 17 IncHI2 plasmids and the R478 reference plasmid were typed by pDLST. This collection was representative of IncHI2 plasmids previously characterized in different countries for the presence of *bla* ($bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}9}$ and $bla_{SHV\text{-}12}$) or *qnr* (*qnrS* and *qnrA*) genes. These plasmids were previously identified in isolates of *E. coli*, *E. cloacae* and *Salmonella* of different serovars obtained from humans, animals and food of animal origin from three continents (Table 3).

By pDLST, 12/17 plasmids were assigned to the ST1 group; the remaining 5/17 plasmids showed the same allele sequence of the pAPEC-O1-R and were assigned to the ST2 group (Table 3). Discrimination among the two major plasmid scaffolds (ST1 and ST2) was also confirmed by RFLP and Southern blot hybridizations experiments (data not shown).

The IncHI2 plasmids carrying the $bla_{CTX\text{-}M\text{-}9}$ gene from *E. coli* and *Salmonella* isolated from 1997 to 2003 in the UK, Spain and France were all assigned to the ST1 group. The $bla_{SHV\text{-}12}$-carrying plasmids, from strains A1 (negative for *qnrA1*) and TcK147 (positive for *qnrA1*), identified in *E. cloacae* and *E. coli* in France and Australia, respectively, were also assigned to the ST1 group. The two IncHI2 plasmids carrying the $bla_{CTX\text{-}M\text{-}2}$ gene and the plasmid from strain 146.71Tc carrying the *qnrS1* gene were classified in the ST2 group, as were the IncHI2

Table 2. Comparison among IncHI2 fully sequenced plasmids

Constant/ variable regions	General functions	IncHI2 plasmid coding sequences				
		R478 NC_005211 (274 762 bp)	pK29 NC_010870 (269 674 bp)	pEC-IMP NC_012555 (318 782 bp)	pEC-IMPQ NC_012556 (324 503 bp)	pAPEC-O1-R NC_009838 (241 387 bp)
C1	replication (HI2), conjugative transfer, tellurite resistance, partition system, surface exclusion, hypothetical proteins, insertion sequences	smr0266-0304 smr0001-0081	p266-310 p001-079	IMP_273-308 IMP_001-074	IMPQ_282-319 IMPQ_001-073	O1R_186-222 O1R_1-74
	target pDLST, *smr0018*; target PBRT, repHI2					
V1	resistance operons and genes: kanamycin, chloramphenicol, mercuric ions, lead, quaternary ammonium, sulphonamides, aminoglycosides, trimethoprim, streptomycin, spectinomycin, nickel, silver, copper, gentamicin, tetracyclines, quinolones (QnrB2) and β-lactams (CMY-8, IMP-8)	smr0082-0159	p080-135	IMP_075-154	IMPQ_074-161	O1R_75-123
	toxin/antitoxin, ABC transporter, hypothetical proteins, insertion sequences, transposons					
	targets HI2 multiplex, *smr0092* and *hipA*					
C2	hypothetical proteins	smr0160-171	p136-146	IMP_155-165	IMPQ_162-172	O1R_124-130
V2	arsenic and aminoglycoside resistance, hypothetical proteins	smr0172-0191	p147-171	IMP_166-182	IMPQ_173-189	O1R_131-134
	target HI2 multiplex, *smr0183*					
C3	UV protection, conjugative transfer system, plasmid inheritance (*hok*), hypothetical proteins, Tn*10*, tetracycline resistance	smr0192-0243A	p172-215	IMP_183-223	IMPQ_190-230	O1R_135-173
	target pDLST, *smr0199*					
V3	resistance operons and genes: chloramphenicol, β-lactams (SHV-12), aminoglycosides, rifampicin, erythromycin and sulphonamides	smr0244-0248	p216-228	IMP_224-262	IMPQ_231-271	absent
	hypothetical proteins, toxin/antitoxin, insertion sequences, transposons					
C4	hypothetical proteins	smr0249-0255	p229-238	IMP_264-269	IMPQ_273-278	O1R_174-180
V4	plasmid maintenance, Lac operon, hypothetical proteins, β-lactams resistance (CTX-M-3), insertion sequences	smr0256-0265	p239-265	IMP_263-272	IMPQ_272-280	O1R_181-185

Table 3. Strains carrying IncHI2 plasmids analysed in this study and results of plasmid typing by pDLST and HI2 multiplex PCR

Sample	Strain	Country	Year	Origin	Fluoroquinolone and β-lactamase resistance genes	Reference	pDLST			HI2 multiplex PCR		
							smr0018	*smr0199*	ST	*hipA*	*smr0092*	*smr0183*
R478	*S. marcescens*	USA	1969	human	neg	3	1	1	1	neg	pos	pos
C4-VLA	*Salmonella* Virchow	UK	1997	human	$bla_{CTX-M-9}$	24	1	1	1	pos	pos	pos
C16-VLA	*Salmonella* Virchow	UK	2003	human	$bla_{CTX-M-9}$	24	1	1	1	pos	pos	pos
1185-DT	*E. coli*	Spain	1998	human	$bla_{CTX-M-9}$	25	1	1	1	pos	pos	pos
1406-DT	*E. coli*	Spain	1999	human	$bla_{CTX-M-9}$	25	1	1	1	pos	pos	pos
C6-VLA	*Salmonella* Virchow	Spain	1997	human	$bla_{CTX-M-9}$	24	1	1	1	pos	pos	pos
C8-VLA	*Salmonella* Virchow	Spain	1999	human	$bla_{CTX-M-9}$	24	1	1	1	neg	neg	pos
C11-VLA	*Salmonella* Virchow	Spain	2001	human	$bla_{CTX-M-9}$	24	1	1	1	pos	pos	pos
112-DT	*Salmonella* Virchow	Spain	2000	human	$bla_{CTX-M-9}$	25	1	1	1	pos	pos	pos
pROU-1	*Salmonella* rough	France	2002	human	$bla_{CTX-M-9}$	11	1	1	1	pos	pos	pos
p3464b-1	*Salmonella* Virchow	France	2003	chicken	$bla_{CTX-M-9}$	11	1	1	1	pos	pos	pos
A1	*E. cloacae*	France	2004	human	bla_{SHV-12}	26	1	1	1	pos	pos	pos
TcK147	*E. coli*	Australia	2002	human	bla_{SHV-12}, *qnrA1*	26	1	1	1	pos	pos	pos
146.71Tc	*Salmonella* Stanley	The Netherlands	2007	human	*qnrS1*, bla_{LAP-2}	5	2	2	2	pos	neg	neg
p142-SA-01-1	*Salmonella* Virchow	Belgium	2001	poultry	$bla_{CTX-M-2}$	21	2	2	2	pos	neg	neg
p03-1902-1	*Salmonella* Virchow	Belgium	2003	poultry	$bla_{CTX-M-2}$	21	2	2	2	pos	neg	neg
17/21	*Salmonella* Saintpaul	Italy	2000	turkey	neg	4	2	2	2	pos	neg	neg
17/24	*Salmonella* Angona	Italy	2000	animal	neg	4	2	2	2	pos	neg	neg
pK29[a]	*K. pneumoniae*	Taiwan	2007	human	$bla_{CTX-M-3}$, bla_{CMY-8}	9	1	1	1	pos	pos	pos
pEC-IMP[a]	*E. cloacae*	Taiwan	2008	human	bla_{IMP-8}, bla_{SHV-12}	10	1	1	1	pos	pos	neg
pEC-IMPQ[a]	*E. cloacae*	Taiwan	2008	human	bla_{IMP-8}, bla_{SHV-12}, *qnrB2*	10	1	1	1	pos	pos	neg
pAPEC-O1-R[a]	*E. coli*	USA	2006	poultry	neg	8	2	2	2	pos	neg	neg

neg, negative; pos, positive.
[a]Results of pDLST and HI2 multiplex PCR as expected by the *in silico* analysis of DNA sequences.

plasmids of the two *Salmonella* strains of animal origin isolated in Italy. Interestingly, four out of five strains of animal origin carried ST2-IncHI2 plasmids; only one carried a ST1-IncHI2 plasmid. The pAPEC-O1-R, prototypic of the ST2 group, was described as a plasmid without quinolone or β-lactamase resistance determinants widely diffused in *E. coli* from poultry in the USA;[8] thus suggesting that ST2-IncHI2 plasmids may prevalently circulate in animal isolates rather than in human isolates.

Using the HI2 multiplex PCR, all the plasmids of the ST1-IncHI2 group except one (C8-VLA) from strains of human origin were PCR positive for the *hipA*/*smr0092*/*smr0183* coding sequences, indicating that they could derive from a common ancestor that is more closely related to pK29 rather than R478 or pEC-IMP (Table 3). In fact, pK29 is the only plasmid among those fully sequenced that showed the presence of the *hipA*, *smr0092* and *smr0183* coding sequences, while the other IncHI2 plasmids showed the deletion of at least one of the three sequences (Table 3). Furthermore, all the plasmids of the ST2 group showed an identical multiplex pattern to that expected for the pAPEC-O1-R, being positive only for the *hipA* region.

We concluded that the IncHI2 plasmids show very well-conserved scaffolds, which belong to two major types: ST1 and ST2. ST1-IncHI2 plasmids similar to pK29 seem prevalently associated with ESBLs such as CTX-M-9 and SHV-12, which show a wide diffusion in human bacterial pathogens.[27–32] In particular, epidemiological studies previously described the spread of the CTX-M-9 variant in Spain as the consequence of the horizontal transfer of a conserved plasmid scaffold circulating among genotypically unrelated bacterial strains.[22,25]

ST2-IncHI2 plasmids, resembling the prototypic avian pAPEC-O1-R plasmid from the USA, were detected in *Salmonella* strains from animals isolated in Europe. In particular, the two plasmids from *Salmonella* Virchow from Belgium associated with the $bla_{CTX\text{-}M\text{-}2}$ gene were categorized as pAPEC-O1-R derivatives. This result is in agreement with the previously described epidemiological association among *Salmonella* Virchow from animals and the increased prevalence of CTX-M-2 in Belgium observed in the recent years.[21] Therefore, plasmid comparison of the IncHI2 backbones contributed to identifying the prevalent mechanism of $bla_{CTX\text{-}M\text{-}2}$ and $bla_{CTX\text{-}M\text{-}9}$ gene diffusion, suggesting that both these genes successfully disseminated in several European countries among different enterobacterial species through the epidemic spread of two major IncHI2 plasmid variants.

The results also suggested that the IncHI2 plasmid family evolves by adding resistance determinants into well-conserved and stable backbones. These plasmids persist stably for years, being disseminated worldwide at very distant geographical sites. Since these plasmids have also been associated with peculiar pili and virulence characteristics, the combination of virulence and resistance may explain their successful spread and stability.

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Transparency declarations

None to declare.

Supplementary data

Figure S1 and Table S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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